Some Observations Concerning Methods for Testing the Resistance of Leather to the Growth of Fungi

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During the war in the South Pacific the prevention of mold on leather became a serious problem. Since there was no standard method for testing the mold resistance of leather and thus evaluating candidate fungicides, a subcommittee† was appointed by the Physical Testing Committee of the American Leather Chemists Association to investigate methods and submit a procedure for approval. The proposed method was approved by the Physical Testing Committee. It has been used by numerous investigators with good results and has been included as one of three acceptable procedures in the Federal Specifications.[3]

Although protecting leather from molds and mildews is no longer so urgent as when the war was raging in the Pacific area, it is still a problem. Even in temperate climates, leather goods are prone to mold during hot humid weather, unless constant care is given them, and in tropical areas the problem is always urgent.

Since there is lack of agreement concerning the efficacy of one test method versus another, it seemed desirable to discuss in detail the Provisional A.L.C.A. method[8] and set forth the reasons for adopting that particular procedure.

Inoculum

The inocula used by various investigators range from single pure cultures to soil suspensions. One method[3] (No. 410.1) employs only a designated strain of *Aspergillus niger,* and the mold resistance of the leather is presumed to be shown by the amount of growth occurring in one week when the sample is incubated on a nutrient agar medium. Several objections to such a method are evident. Fungicides are notoriously selective in their action on molds. A certain material may completely inhibit the growth of one or several organisms and yet be completely ineffective against other species or even different strains of the same species. Furthermore, although *Aspergillus niger* almost always grows luxuriantly on vegetable tanned leathers under favorable conditions, it does not grow well on some other types of leather, for instance, straight chrome tanned leather, and frequently cannot compete

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† The subcommittee consisted of Fred O'Flaherty, Tanners' Council Laboratory, and J. S. Rogers, Eastern Regional Research Laboratory.

with other species.* It would seem then that this method determines only the resistance of leather to the growth of *Aspergillus niger* under certain specified conditions and is of little significance in determining its resistance under actual conditions of use.

Another method[9] advocated by some workers utilizes one known strain of *Aspergillus niger* and an unidentified species of *Penicillium* for inoculation. This method is subject to essentially the same limitations as the one just discussed, that is, inadequacy of the inoculum.

A method[3] (No. 412.1) which employs "a moist soil rich in microbial life," enriched with *Metarrhizium glutinosum* and *Aspergillus ustus* spores as the inoculum would be very difficult to standardize. Soils vary greatly in regard to reaction, organic matter content, etc., and the phrase "rich in microbial life" has no definite meaning. A soil might contain a few types of micro-organisms, for example, algae or anaerobic bacteria, to the virtual exclusion of molds and mildews and yet fit the phrase perfectly. Furthermore, the immersion of test specimens in a soil suspension would create conditions far different from those found in actual practice. Most of the molding of leather articles in tropical or semi-tropical climate occurs during storage, where there is little chance of contamination with nutrient materials or of inoculation by other than air-borne organisms.

In this connection the often suggested soil burial tests should be mentioned. Although such tests undoubtedly serve a useful purpose for testing the resistance to deterioration of many textiles, they would seem to have little or no place in testing leather. Only shoes are continually exposed to soil, and even they are not exposed to burial conditions. As just pointed out, it is primarily the undisturbed exposure of leather to dark, humid conditions which promotes mold growth; leather in active use is seldom attacked. Soil burial conditions have but slight similarity to the condition to which leather goods are subjected, and therefore need not be considered.

A mixed, mass inoculum, such as that employed in the A.L.C.A. and other[1] methods has the advantage of approximating conditions in nature, where mixed cultures are always the rule. The problem of maintaining a large number of pure cultures has been circumvented by using a mixture of the spores of pure cultures, dried and mixed with sterile sand. To prepare this inoculum the molds, in pure culture, are grown on a nutrient broth or agar medium until abundant sporulation has taken place. The growths are removed, and then if they are grown on a liquid medium, the free moisture is pressed out, and they are dried for 5 to 10 days at 35° C. The dried mats are ground in a mortar with iron-free sand and thoroughly mixed together. Strict aseptic precautions are not necessary during the drying and grinding operations, since the leather samples to be treated are not sterile and the

* Unpublished data obtained by the author.

small amount of contamination is insignificant, as compared with the mass of spores present. This procedure completely obviates the often heard objection to mass inoculation, namely, that certain organisms may inhibit others, for if growth occurs on a specimen, lack of mold resistance is shown regardless of the species that develop.

Except for Nos. 1 and 12, the molds in the mixture were isolated from moldy leather from the South Pacific or from domestic samples, and all have grown luxuriantly on one or more types of leather. The identity* of the cultures included in the mixture follows:

A.L.C.A.	No. 1	*Aspergillus repens*
"	No. 2	*Paecilomyces varioti* (typical strain)
"	No. 3	*Rhizopus arrhizus*
"	No. 4	*Penicillium namylowskii* (appears to be a strain of)
"	No. 5	*Aspergillus niger* (typical strain)
"	No. 6	*Aspergillus fumigatus*
"	No. 7	A member of the *Penicillium spinulosum* series†
"	No. 8	*Aspergillus terreus*
"	No. 9	*Penicillium oxalicum* (typical strain)
"	No. 10	*Penicillium luteum* (a nonascosporic strain)
"	No. 11	A member of the *Penicillium funiculosum* series
"	No. 12	*Metarrhizium glutinosum*
"	No. 13	*Aspergillus flavus* (typical strain)
"	No. 14	*Gliocladium fimbriatum*

Mold cultures prepared in this way remain viable for a long time. One mixture, stored in a refrigerator, was still viable after 28 months. The viability of individual molds after storage was tested by preparing cultures in the usual manner but keeping them separate. Only one culture now included in the Official mixture died during 20 months' storage at room temperature. Although four cultures now in the Official mixture were not included in this test, it seems certain that all of them will remain viable for the specified 6 month period during which the Official preparations are valid. The specific cultures are purposely not listed in the method, so that additions or changes can be made as needed without approval of the society.

The advantages of such a preparation are obvious. It is standard; there is no need for maintaining pure cultures; no equipment for preparing or sterilizing media is necessary; and it can be used much as a reagent is, by those who do not have training in bacteriological technique.

* Dr. Kenneth B. Raper of the Northern Regional Research Laboratory, Peoria, Ill., kindly identified all the cultures except Nos. 1 and 12, which were already known.

† A strain somewhat atypical of the series, heavier sporing than most, and producing very long, thin, columns of spores on some culture media.

TABLE I

COMPOSITION OF MEDIA

Component	Gelatin-Czapek* Grams per liter	Milk-Agar† Grams per liter	Casein-Czapek-Agar‡ Grams per liter
Gelatin (Difco Bacto-Gelatin)	100.0	....	....
Fresh skim milk (centrifuged)	....	500.0	....
Casein (Coleman and Bell C.P.)	....	....	50.0
Agar (Difco Bacto-Agar)	....	15.0	15.0
Sucrose	30.0	....	30.0
Ammonium sulfate	2.0	....	2.0
Di-potassium acid phosphate	1.0	....	1.0
Potassium chloride	0.75	....	0.75
Magnesium sulfate (hydrated)	0.50	....	0.50
Ferrous sulfate	0.01	....	0.01
Water	q.s.	q.s.	q.s.

*Sterilized at 10 pounds steam pressure per square inch for 30 minutes.
†Sterilized at 10 pounds' steam pressure per square inch for 20 minutes.
‡Sterilized at 15 pounds' steam pressure per square inch for 20 minutes.

*Growth Characteristics of the Organisms**

Since these organisms were originally isolated from various naturally contaminated leathers or similar materials, it was felt desirable to determine some of their growth characteristics. Because it was known that these characteristics would be influenced by the composition of the growth medium, three media—gelatin-Czapek, milk agar and casein-Czapek-agar were selected for study. Table I shows their compositions. One medium contains gelatin, a protein related to skin collagen; the other two media contain other proteins. Milk-agar is a relatively unaltered natural medium, whereas casein-Czapek is an artificial medium containing casein, a denatured milk protein. This protein is sometimes used in leather finishes, and therefore might be a contributing factor to the growth of molds on leather.

The growth of these organisms was observed in Petri dishes. The size of the colonies was measured periodically over a period of 18 days. Visual examination showed other growth characteristics. Figures I, II, III, and IV show the growth curves of these organisms. The maximum growth area was 63.6 square centimeters, the area of the Petri dish.

A.L.C.A. Mold No. 1 grew fairly well on the casein-Czapek-agar medium, with proteolytic activity, indicated by clearing of the media, starting on the sixth day. This clearing extended in a narrow fringe outside the area of growth as well as under the colony area. On milk-agar, on the other hand, growth was much poorer, with almost no clearing. There was no liquefaction of gelatin during the entire 18 day period of slow growth. Sporulation was fairly good on all three media.

* This section was contributed by R. M. Lollar of the Tanners' Council Research Laboratory, University of Cincinnati.

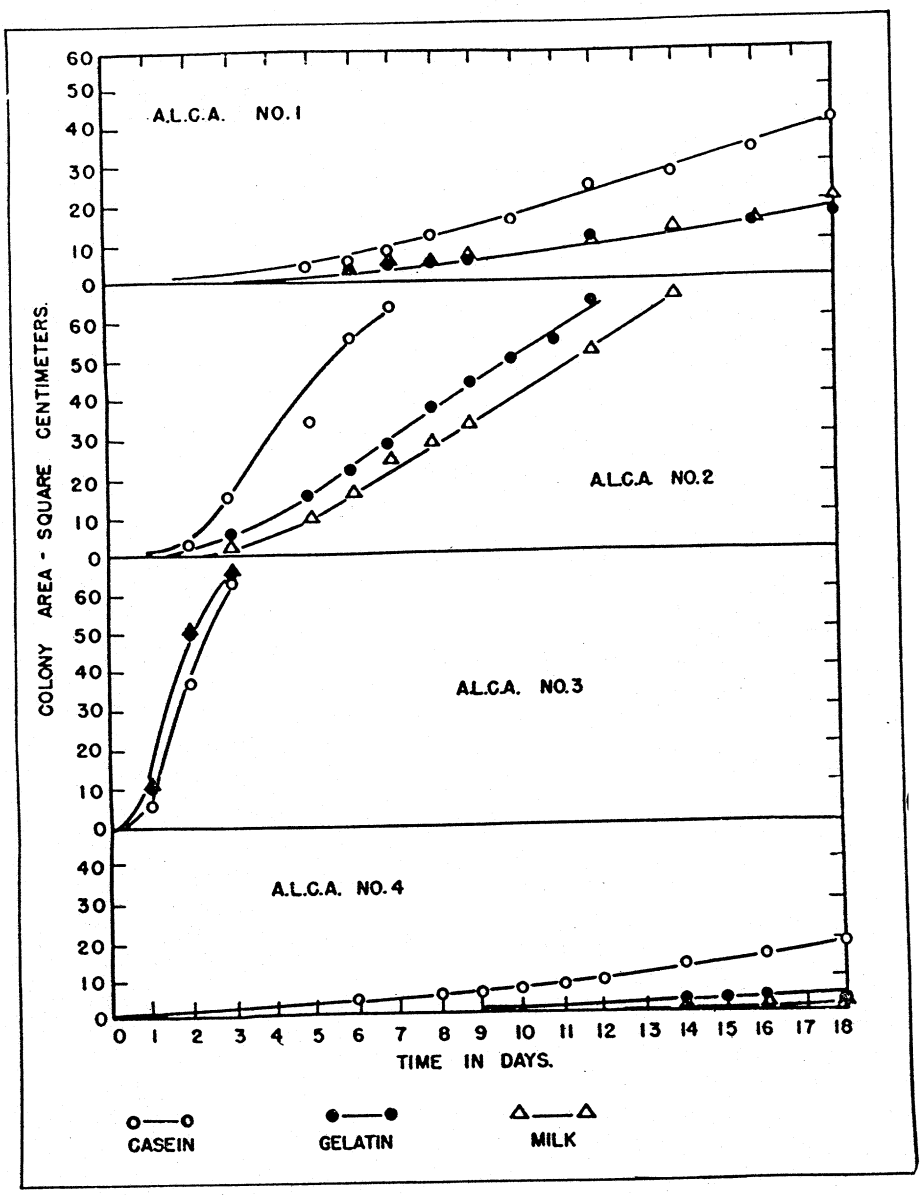

FIGURE I. Growth Rates for Molds 1, 2, 3, and 4 on Different Media.

A.L.C.A. Mold No. 2 grew appreciably better than Mold No. 1 on all three media. On casein, the growth spread rapidly, the colony color remained avellaneous, and sporulation was excellent. The medium cleared under the colony, starting about the third day. The growth on milk-agar was much

poorer, with no observable clearing. The gelatin-Czapek medium was not so good a growth medium as casein, but it did support good growth, with liquefaction, which started on the sixth day.

A.L.C.A. Mold No. 3 grew the most rapidly of all the molds, and proteolytic activity was evident on all three media. In contrast, A.L.C.A. Mold

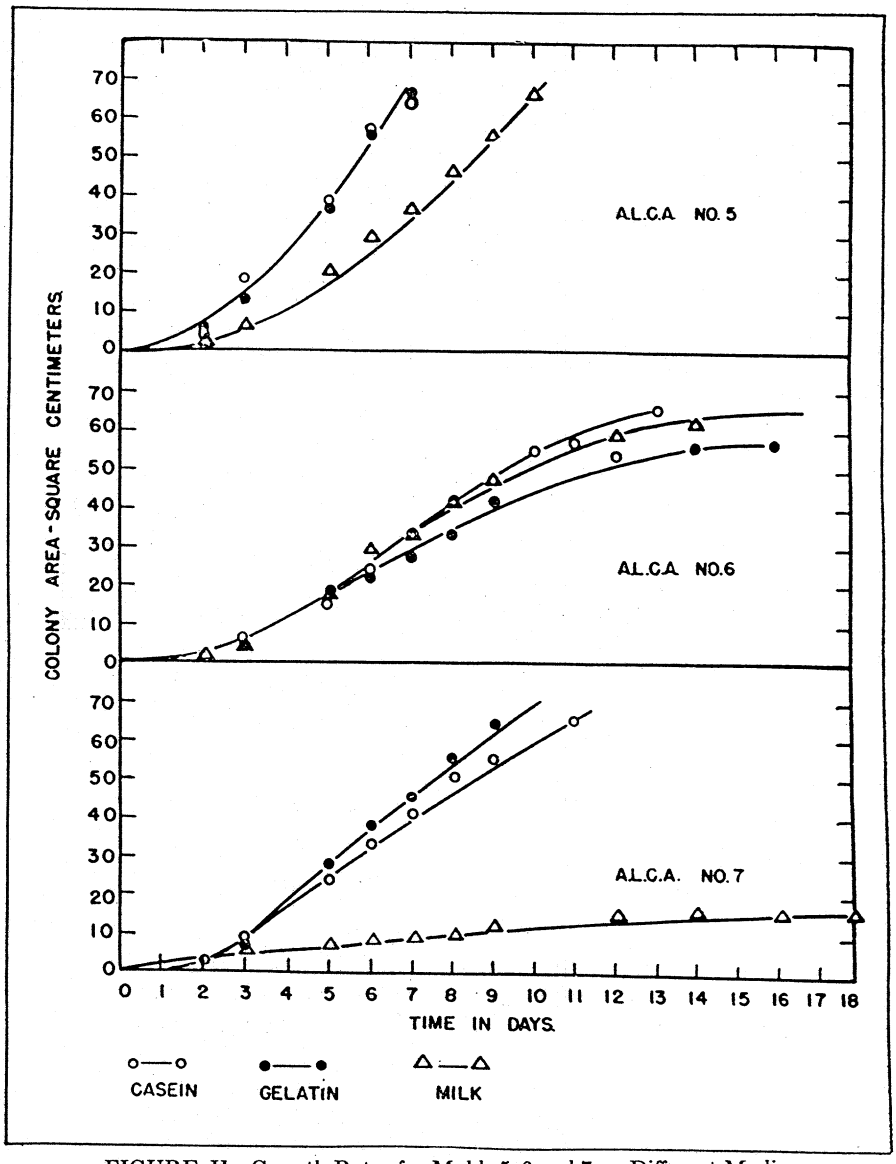

FIGURE II. Growth Rates for Molds 5, 6 and 7 on Different Media.

No. 4 grew slowly on all three media, though the greenish sporulation was good. Number 4 showed proteolytic activity only on casein.

A.L.C.A. Mold No. 5 grew luxuriantly, with moderate spreading on all three media. Heavy black sporulation was also noted. Proteolytic activity was marked on all three media.

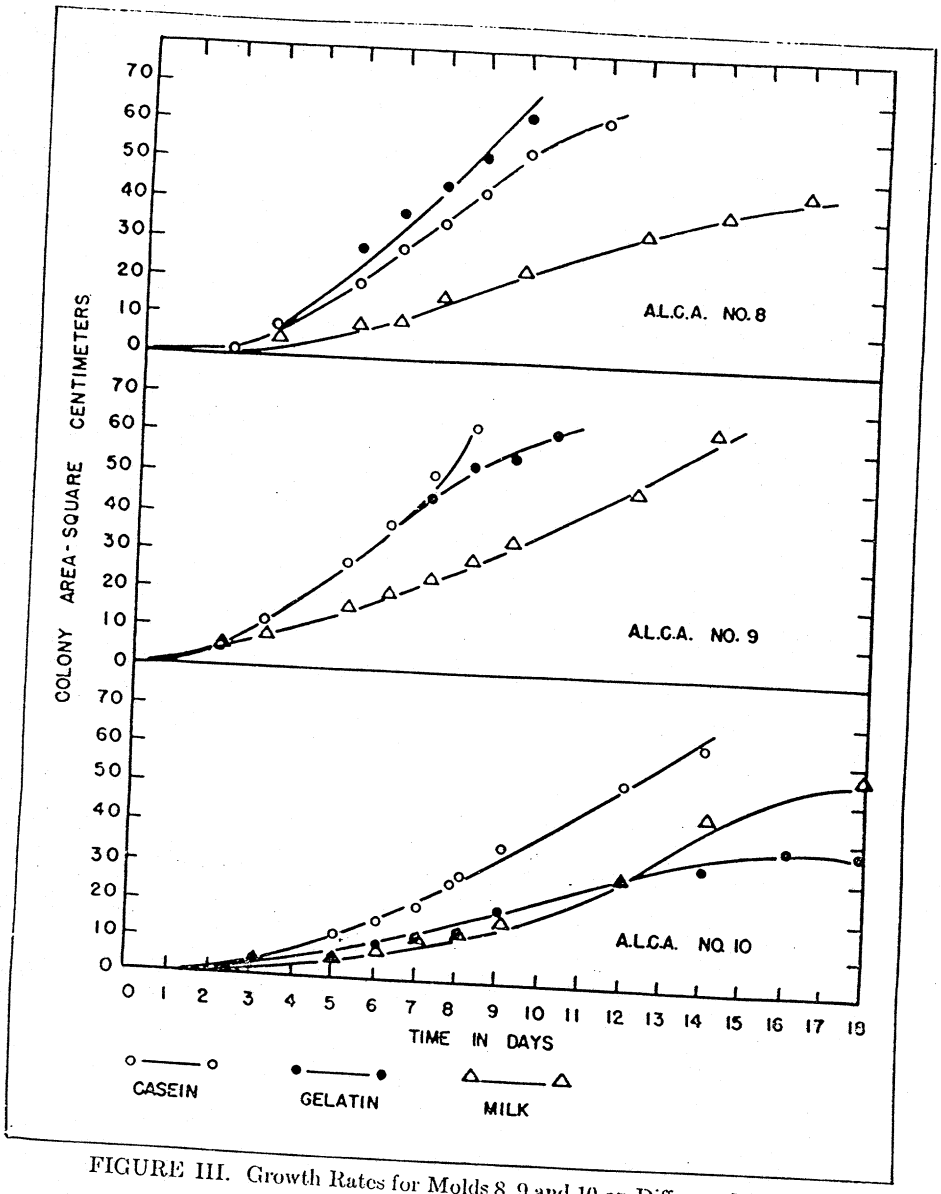

FIGURE III. Growth Rates for Molds 8, 9 and 10 on Different Media.

A.L.C.A. Mold No. 6 grew well on all the media, although the development of spores on milk-agar was slow. Clearing of the casein and milk and liquefaction of gelatin in the first few days of growth attested to its proteolytic activity.

A.L.C.A. Mold No. 7 was characterized by the wide difference in its growth on gelatin and casein in contrast to that on milk. On gelatin-Czapek medium and casein-Czapek-agar medium, growth was good, sporulation was heavy, and proteolytic activity was noted in three to five days. In contrast, on milk-agar, growth and sporulation were poor, and no proteolysis was noted.

A.L.C.A. No. 8 and No. 9 showed rather similar growth characteristics on the three media. No. 9 showed more evidence of proteolytic activity, having marginal areas of clearing around the growth area.

A.L.C.A. No. 10 and No. 11 both grew rather slowly on all three media. A.L.C.A. No. 10 finally overgrew the area of the dish on casein, but like No. 11 on all media, on the other two media it was not able to spread over the entire dish area. Sporulation and proteolytic activity were good on all media for both molds. A.L.C.A. Mold No. 12 was an American Type Culture Collection organism, *Metarrhizium glutinosum,* which replaced a poor growing *Metarrhizium* which had been included originally. Since this change was made after these evaluations were completed, the characteristics of No. 12 were not determined.

A.L.C.A. Mold No. 13 was characterized by poor growth on milk-agar. The greenish-yellow conidial heads on this medium were conspicuously arranged in a series of concentric zones.

Like No. 3, A.L.C.A. Mold No. 14 grew rapidly on all three media. Sporulation and proteolytic activity were also high.

Procedure

Preparation for Inoculation

Washing is prescribed to remove water soluble fungicides, which are valueless for most purposes. Leaching with running water might be satisfactory, but would not be so effective as shaking or drumming and would be more time consuming. In some cases special leaching procedures might be required. For example, in testing leather for some articles to be used on ship board it would be advisable to subject the specimens to the action of salt water of about the same concentration as sea water. In other cases leaching or washing may not be needed at all. Some leather goods are never subjected to the action of water and a water-soluble fungicide might serve the purpose very well. The option of washing only the specimens which have shown resistance to mold growth when not washed is included so that the additional information may be obtained if desired.

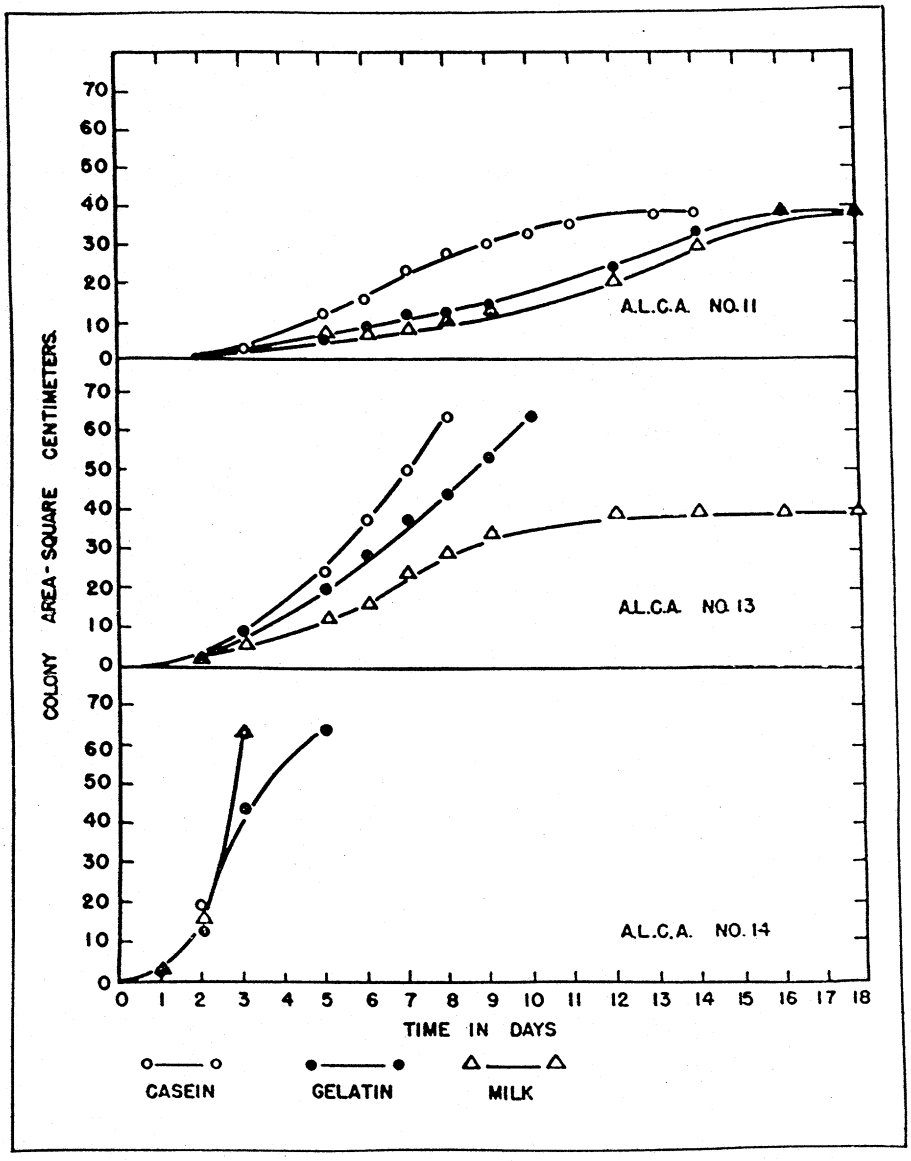

FIGURE IV. Growth Rates for Molds 11, 13 and 14 on Different Media.

Inoculation

When the Official spore mixture is used, inoculation is an extremely simple operation. Some workers prefer using a filtered water suspension to dusting. They claim that small particles of sand left on the specimen by the latter procedure may be mistaken for mold growth. Specimens should not be dipped

in a spore suspension. Enough fungicide might be washed out to alter the viability of the spores.

Incubation

At least four factors under this heading need consideration. They are temperature, humidity, time, and the type of incubation chamber.

Temperature. The mold fungi show a wide range of temperature optima. However, those included in the mixture are not particularly fastidious and would probably grow well at any temperature between about 20° and 35° C. If no controlled temperature device is available, ordinary laboratory temperatures would probably prove satisfactory.

Humidity. It has been established that most mold fungi will not germinate and grow below relative humidities of about 70 to 75 per cent.[4,5] It is therefore necessary to maintain the humidity of the incubation chamber above this figure and for accelerated growth it should be kept above 85 per cent.

Time. The time required for mold to develop on different leathers varies greatly. A vegetable tanned sole leather, loaded with sugar, will often mold in 24 hours under the conditions of the test. On the other hand alum retanned vegetable leather may remain mold-free for several weeks. Leathers treated with candidate fungicides are even more likely to show wide variations.[7] To be of value, mold resistance must have some degree of permanence. Many test methods which seem to produce drastic acceleration, such as those in which the specimens are placed on a nutrient agar medium or solution, do not give a true picture of what is likely to happen in practice and may lead to entirely erroneous conclusions. Under such conditions a sample might well mold; whereas in actual practice it would be completely protected. It seems justifiable, then, to prescribe an incubation period of at least 1 month, and where practicable the period might even be extended.

Type of Incubation Chamber. At first thought it would appear that any chamber, cabinet, etc., in which the proper temperature and humidity could be maintained would be satisfactory. Some fungicides volatilize, however, and the toxic vapors, if confined, may build up to a high enough concentration to inhibit growth on specimens treated with other materials or even on the untreated controls. Hence the stipulation in the A.L.C.A. method that "each fungicidal treatment shall be incubated in a separate compartment." At this laboratory we have used 8 or 12 oz. wide-mouth jars with plastic caps. Small holes are drilled in opposite sides of the cap, and heavy cord or rustproof wire is threaded through the holes and tied. Small hooks, made from 1 or 2 mm. glass rod are used to suspend the specimens from the wire or cord. Each jar accommodates 4 to 6 specimens.

It is possible that volatility of fungicides might also cause difficulty in this set-up. It is conceivable that toxic vapors might prevent mold growth

in a confined space, whereas under conditions of use they would be dissipated. We are contemplating the construction of a cabinet in which air would be continuously exhausted and replaced with humidified air, or of a manifold to accomplish the same effect with the jars.

Results

Although physical damage to leather by molds has been reported,[2,6] it is usually too slight to be used as a criterion for extent of damage. Discolorations, mustiness, stiffness, etc., are not produced regularly and are also not suitable as a measure of deterioration. However, the visual observation of mold or absence of mold on the specimens is sufficient for determining results of the test. More detailed information can be obtained if specimens are examined at frequent intervals and a record is made of the time of first appearance and the approximate extent of growth. A low-power microscope or good hand lens aids in detecting the first appearance of growth and in settling doubtful cases, but it is not absolutely essential.

Summary

The proposed A.L.C.A. method for testing the resistance of leather to mold growth is discussed, and reasons are given for each step in the procedure. The advantages of the method, namely, that it duplicates essential conditions of natural exposure and can be used with a minimum of laboratory equipment and mycological training, are pointed out.

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